

Year	Agency	Carcinogenicity Classification	Findings
2004 <sup>d</sup>	IARC (Monographs Volume 88, 2006)	Carcinogenic to humans (Group 1)	<b>Epidemiological evidence.</b> Sufficient, based on nasopharyngeal cancer
			Leukemia: "There is strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to formaldehyde. Increased risk for leukaemia has consistently been observed in studies of professional workers and in two of three of the most relevant studies of industrial workers. These findings fall slightly short of being fully persuasive because of some limitations in the findings from the cohorts of industrial and garment workers in the USA and because they conflict with the non-positive findings from the British cohort of industrial workers." (p.276)
			<b>Toxicological evidence.</b> Sufficient (nasal squamous cell carcinoma)
			<b>Supporting data.</b> Mechanism for inducing myeloid leukemia is not known. Possible mechanisms considered included clastogenic damage to circulatory stem cells.  "The Working Group was not aware of any good rodent models that simulate the occurrence of acute myeloid leukaemia in humans. Therefore, on the basis of the data available at this time, it was not possible to identify a mechanism for the induction of myeloid leukaemia in humans." (p. 280)
2009 <sup>e</sup>	IARC (Monographs Volume 100F, 2012)	Carcinogenic to humans (Group 1)	<b>Epidemiological evidence.</b> Formaldehyde causes cancer of the nasopharynx and leukaemia.
			"The Working Group was not in full agreement on the evaluation of formaldehyde causing leukaemia in humans, with a small majority viewing the evidence as sufficient of carcinogenicity and the minority viewing the evidence as limited." (p. 430)
			<b>Toxicological evidence.</b> "Studies of bone marrow cells in formaldehyde-exposed animals have been inconsistent." (p.427) "Pancytopenia has not been among the haematological findings in experiments with laboratory animals exposed to relatively high doses of formaldehyde, including classic long-term safety assessment studies." (p.428) Inconsistent genotoxic effects in blood lymphocytes from animals exposed to formaldehyde via inhalation.
			<b>Supporting data.</b> "Particularly relevant to the discussions regarding sufficient evidence was a recent study accepted for publication which, for the first time, reported aneuploidy in blood of exposed workers characteristic of myeloid leukaemia and myelodysplastic syndromes, with supporting information suggesting a decreased in the major circulating blood-cell types and in circulating haematological precursor cells. The authors and Working Group felt that this study needed to be replicated." (p. 430) "Three possible mechanisms, all focused around genotoxicity, are moderately supported as the underlying mechanism for induction of haematological malignancies in humans. Further research is needed to decide which of the mechanisms is the most important." (p. 430)

Year	Agency	Carcinogenicity Classification	Findings
2010	EPA (Draft IRIS Toxicological Review, 2010)	Carcinogenic to humans	<p><b>Epidemiological evidence.</b> Sufficient</p> <p>“Human epidemiological evidence is sufficient to conclude a causal association between formaldehyde exposure and nasopharyngeal cancer, nasal and paranasal cancer, all leukemias, ML and lymphohematopoietic cancers as a group” (page 6-46).</p> <p>For all LHM combined: “Given the consistency and strength of the positive associations for all LHP [lymphohematopoietic] cancer mortality in professional cohorts (embalmers, anatomists and pathologists) taken together with the strong positive results of the NCI cohort, human epidemiologic evidence are [sic] sufficient to conclude that there is a causal association between formaldehyde exposure and mortality from all LHP malignancies (as a group.)” (page 4-180).</p> <p>For all leukemias as a group: “While the epidemiologic evidence for a causal association between formaldehyde and all leukemia as a group is not at [sic] strong as for all LHP as a group, the repeated identification of an association in multiple meta-analyses taken together with the clear causal association between myeloid leukemia demonstrated by Hauptmann et al. (2009) and the consistent evidence reported by Beane Freeman et al. (2009) are sufficient to conclude that there is a causal association between formaldehyde exposure and mortality from all leukemia as a group.” (page 4-182)</p> <p><b>Toxicological evidence.</b> Limited evidence to support conclusion that formaldehyde exposure causes leukemia. Four studies evaluated the leukemic potential of formaldehyde.</p> <p>“Inhalation exposure of formaldehyde increased lymphoma in female mice and leukemia in female F344 rats, but not male rats (Battelle Laboratories, 1981). No increases in leukemia or lymphoma were seen in male Wistar rats when exposed to formaldehyde in drinking water (Til et al., 1989) or male rats after chronic inhalation exposures (Sellakumar et al., 1985).” (p.6-21)</p> <p><b>Supporting data.</b></p> <p>“Chromosomal damage in blood-borne immune cells, relevant to agent-induced lymphohematopoietic cancers has been documented in formaldehyde exposed workers, including increased micronuclei and chromosomal aberrations, increased incidence and aneuploidy in hematopoietic stem cells.” (p.6-22)</p>
2012	NTP (12 <sup>th</sup> RoC, 2013)	Known to be a human carcinogen	<p><b>Epidemiological evidence.</b> Causes nasopharyngeal cancer, sinonasal cancer, and myeloid leukemia</p> <p>“Epidemiological studies have demonstrated a causal relationship between exposure to formaldehyde and cancer in humans. Causality is indicated by consistent findings of increased risks of nasopharyngeal cancer, sinonasal cancer, and lymphohematopoietic cancer, specifically myeloid leukemia among individuals with higher measures of exposure to formaldehyde (exposure level or duration), which cannot be explained by chance, bias, or confounding. The evidence for nasopharyngeal cancer is somewhat stronger than that for myeloid leukemia.” (p. 195)</p> <p><b>Toxicological evidence.</b> No specific evidence cited regarding leukemia beyond the following:</p> <p>“Hemolymphoreticular tumor (combined types) in rats of both sexes also were significantly increased</p>

Year	Agency	Carcinogenicity Classification	Findings
			<p>after long-term exposure of adults; however, it is unclear whether these tumors were exposure-related, because of limitations in the reporting of these tumors (Soffritti et al., 2002)." (p. 198)</p> <p><b>Supporting data.</b>          "Lymphohematopoietic cancers are a heterogeneous group of cancers that arise from damage to stem cells during hematopoietic and lymphoid development (Greaves 2004). Blood cells arise from a common stem cell, which forms two progenitor cells, the common myeloid stem cell and the common lymphoid stem cell. Most agents known to cause leukemia are thought to do so by directly damaging stem cells in the bone marrow. In order for a stem cell to become malignant, it must acquire genetic mutations and genomic instability (Zhang et al. 2010a). Because formaldehyde is highly reactive and rapidly metabolized, a key question is how it can reach the bone marrow or cause toxicity or genotoxicity at distal sites. The endogenous concentration in the blood of humans, monkeys, and rats is about 2 to 3 µg/g, and the concentration does not increase after inhalation of formaldehyde from exogenous sources (Heck et al. 1985, Casanova et al. 1988, Heck and Casanova et al. 2004). Moreover, N2-hydroxymethyl-dG-DNA adducts have not been detected at distal sites in rats (such as the bone marrow, white blood cells, lung, spleen, liver, or thymus) (Lu et al. 2010). For these reasons, the plausibility of formaldehyde's causing cancer at distal sites, such as myeloid leukemia, has been questioned (Golden et al. 2006, Pyatt et al. 2008).          However, systemic effects have been observed after inhalation or oral exposure, and although the mechanisms by which formaldehyde causes myeloid leukemia in humans are not known, a number of plausible mechanisms have been advanced. These include (1) theoretical mechanisms for the distribution of formaldehyde to distal sites and (2) proposed mechanisms of leukemogenesis that do not require formaldehyde to reach the bone marrow. In addition, there is some evidence that formaldehyde causes adverse haematological effects in humans." (p. 199)</p>
2012	RAC (RAC, ECHA, 2012)	Carc. 1B - H50 <sup>f</sup> May cause cancer	<p><b>Epidemiological evidence.</b> Limited          "In conclusion, while some studies have found increased rates of leukaemia, the epidemiology data do not show consistent findings across studies for leukaemia rates. The inconsistent findings across job types and exposure groupings, and the lack of biological plausibility argue against formaldehyde as the cause of the increased rates. The findings of slightly increased leukaemia rates among embalmers, pathologist and anatomists, but not among industrial workers, suggests the possibility of confounding factors that bear investigation. Results based on cohort and case-control studies do not suggest an association between formaldehyde exposure and leukaemia." (p.41)</p> <p><b>Toxicological evidence.</b> "No indication of carcinogenic potential on organs/tissues distant from the site of contact (respiratory tract) including lymphohaematopoietic tumours in inhalation study of rats and mice (Kerns et al. 1983)." (p.22)</p> <p><b>Supporting data.</b> "Physiologically, formaldehyde occurs in most organisms, tissues and cells at very low concentrations. In mammals, formaldehyde is found at values of about 0.1 mM in blood (man, monkey, rat). The physiological blood formaldehyde levels in humans, rats and monkeys were not elevated after parenteral exposure, indicating a very low systemic tissue and organ distribution of formaldehyde.</p>

Year	Agency	Carcinogenicity Classification	Findings
			These findings support evidence that formaldehyde shows local reactivity and elicits its toxic potential focally and predominantly at deposition areas such as epithelia of the upper respiratory tract, the orogastric tract as well as the skin. (BfR-Wissenschaft, 2006). Thus, it may be expected that carcinogenic effects are not found at anatomical sites distant from the port of entry." (p.44)
2016	Scientific Committee on Occupational Exposure Limits for Formaldehyde (SCOEL, 2016)	Carcinogen Group C (genotoxic carcinogen with a mode-of-action based threshold)	<p><b>Epidemiological evidence.</b> "A possible induction of myeloid leukaemias by FA in humans is not so easy to explain, but there are indications that FA might induce this kind of malignancy. However, this would require that FA would act systemically and reach the bone marrow, which is the target tissue. Such an action would not be possible within a range where the external dose does not change the physiological level of FA." (p.45)</p> <p><b>Toxicological Evidence.</b> "In essence, new experimental data, reported since 2008, clearly indicate that systemic genotoxic action of inhaled FA is not likely, even at exposure concentrations leading to nasal malignancies in the rat." (p.49)</p> <p><b>Supporting Data.</b> "A plethora of arguments suggests that FA concentrations below 1 or 2 ppm would not increase the risk of cancer in the nose or any other tissue, or affect FA homeostasis within epithelial cells (Swenberg et al., 2013)." (p. 49)</p>

<sup>a</sup>IARC Working Group met February 1981. IARC Preamble (1982): "For many of the chemicals evaluated in the first 29 volumes of the IARC Monographs for which there is sufficient evidence of carcinogenicity in animals, data relating to carcinogenicity for humans are either insufficient or nonexistent. In the absence of adequate data on humans, it is reasonable, for practical purposes, to regard chemicals for which there is sufficient evidence of carcinogenicity in animals as if they presented a carcinogenic risk to humans. The use of the expressions 'for practical purposes' and 'as if they presented a carcinogenic risk' indicates that at the present time a correlation between carcinogenicity in animals and possible human risk cannot be made on a purely scientific basis, but only pragmatically. Such a pragmatical correlation may be useful to regulatory agencies in making decisions related to the primary prevention of cancer."

<sup>b</sup>IARC Working Group met March 1987.

<sup>c</sup>IARC Working Group met October 1994; monograph published 1995.

<sup>d</sup>IARC Working Group met June 2004; monograph published 2006.

<sup>e</sup>IARC Working Group met October 2009; monograph published 2012.

<sup>f</sup>EU harmonized classification and labelling.

Table 2: Summary of NAS (2011) Comments or Identified Data Gaps and New Formaldehyde Science by Lines of Inquiry

NAS (2011) Comment / Identified Data Gap	New Formaldehyde Science
<b>A. Epidemiological Evidence</b>	
Evaluation of the most specific diagnoses available in the epidemiologic data (i.e., acute myeloblastic leukemia, chronic lymphocytic leukemia, and other specific lymphomas). (NAS, p. 113)	<p>New analyses of the NCI formaldehyde workers cohort specifically for AML are reported. Results do not support the hypothesis that formaldehyde causes AML. <b>Checkoway et al. (2015)</b></p> <p>Associations seen between formaldehyde exposure and Hodgkin lymphoma and chronic myeloid leukemia (CML) have not been observed in other studies and are not considered plausible. <b>Checkoway et al. (2015)</b></p>
Because the draft IRIS assessment relies solely on epidemiologic studies to determine causality, further discussion of the specific strengths, weaknesses, and inconsistencies in several key studies is needed. (NAS, p. 113)	<p>A critical review of the epidemiological literature indicated no consistent or strong epidemiologic evidence that formaldehyde is causally related to any lymphohematopoietic malignancies. The absence of established toxicological mechanisms further weakens any arguments for causation. <b>Checkoway et al. (2012)</b></p>
Clarification of the basis of its interpretations of the results regarding the various dose metrics (peak versus cumulative) and the various LHP cancers. (NAS, p. 112-113)	<p>Acute myeloid leukemia (AML) was unrelated to cumulative, average or peak exposure, and few deaths occurred within 20 or more years of last peak exposure. Suggestive associations with peak exposure were observed for chronic myeloid leukemia, based on very small numbers. Hodgkin lymphoma relative risk estimates suggested trends for both cumulative (<math>p_{\text{trend}} = 0.05</math>) and peak (<math>p_{\text{trend}} = 0.003</math>) exposures. However, no other lymphohematopoietic malignancy was associated with either cumulative or peak exposure. <b>Checkoway et al. (2015)</b></p>
The selection and use of the NCI cohort (Beane-Freeman et al. 2009) should be further justified. (NAS, p. 112)	<p>Extended follow-up of a cohort of 14,008 chemical workers at 6 factories in England and Wales, covering the period 1941-2012. Results provide no support for an increased hazard of myeloid leukemia from formaldehyde exposure. <b>Coggon et al. (2014)</b></p>

NAS (2011) Comment / Identified Data Gap	New Formaldehyde Science
	<p>Extended follow-up of 11,098 employees of three garment manufacturing facilities. Results demonstrated limited evidence for formaldehyde exposure and any LHM including AML, based on 14 observed cases.</p> <p><b>Meyers et al. (2013)</b></p>
<b>B. Toxicological Evidence</b>	
<p>Paucity of evidence of formaldehyde-induced LHP cancers in animal models. EPA's unpublished re-analysis of the Battelle chronic experiments in mice and rats (Battelle Columbus Laboratories 1981), although intriguing, provides the only positive findings and thus does not contribute to the weight of evidence of causality. (NAS, p. 110)</p>	<p>No cases of leukemia or lymphohematopoietic neoplasia were seen. FA inhalation did not cause leukemia in genetically predisposed C3B6.129F1-Trp53tm1Brd mice.</p> <p><b>Morgan et al. (2014)</b></p>
	<p>FA inhalation did not cause leukemia or lymphohematopoietic neoplasia in genetically predisposed p53-Haploinsufficient mice. <b>Morgan et al. (2015)</b></p>
<b>C. Mode of Action Evidence</b>	
<p>Improve understanding of when exogenous formaldehyde exposure appreciably alters normal endogenous formaldehyde concentrations. (NAS, p. 58)</p>	<p>Endogenous formaldehyde in nasal tissues did not significantly affect flux or nasal uptake predictions at exposure concentrations &gt; 500 ppb; however, reduced nasal uptake was predicted at lower exposure concentrations.</p> <p><b>Schroeter et al. (2014)</b></p>
	<p>With the application of highly sensitive instruments and accurate assays, inhaled formaldehyde was found to reach nasal respiratory epithelium, but not other tissues distant to the site of initial contact. In contrast, endogenous adducts were readily detected in all tissues examined with remarkably higher amounts present. Moreover, the amounts of exogenous formaldehyde-induced adducts were 3- to 8-fold and 5- to 11-fold lower than the average amounts of endogenous formaldehyde-induced adducts in rat and monkey nasal respiratory epithelium, respectively.</p> <p><b>Yu et al. (2015)</b></p>

NAS (2011) Comment / Identified Data Gap	New Formaldehyde Science
<p>Reconcile divergent statements regarding systemic delivery of formaldehyde (<i>p.59</i>); direct evidence of systemic delivery of formaldehyde is generally lacking. (<i>NAS, p.5</i>)</p>	<p>Based on a sensitive analytical method that can measure endogenous versus exogenous formaldehyde DNA adducts, the multiple studies demonstrated that inhaled exogenous formaldehyde only reached rat or monkey noses, but not tissues distant to the site of initial contact. Also, new evidence suggests that endogenous formaldehyde in bone marrow is toxic and carcinogenic, and may cause leukemia (but not exogenous formaldehyde).  <b>Lai et al. (2016)</b>  <b>Gao et al. (2016)</b>  <b>Yu et al. (2015)</b>  <b>Edrissi et al. (2013)</b>  <b>Moeller et al. (2011)</b>  <b>Lu et al. (2011)</b></p>
<p>Data are insufficient to conclude definitively that formaldehyde is causing cytogenetic effects at distant sites. (<i>NAS, p. 5</i>)</p>	<p>Critical review of the genotoxicity literature found no convincing evidence that exogenous exposures to FA alone, and by inhalation, induce mutations at sites distant from the portal of entry tissue as a direct DNA reactive mutagenic effect – specifically not in the bone marrow.</p> <p>Review of the existing studies of hematotoxicity, likewise, failed to demonstrate myelotoxicity in any species– a probable prerequisite for leukemogenesis.  <b>Albertini and Kaden (2016)</b></p> <p>Reanalysis of selected raw data from the Zhang et al. (2010) study do not support a causal association between formaldehyde and myeloid leukemia or lymphoid malignancies. Because of the significant methodological limitations, unless the results can be confirmed using appropriate methodologies designed to detect in vivo events, the reanalysis of the results provided by Zhang et al. (2010) raise sufficient questions that limit the use of Zhang et al. (2010) to support the hypothesis that formaldehyde exposure is causally related to leukemia or lymphoid malignancies.  <b>Gentry et al. (2013)</b></p>

NAS (2011) Comment / Identified Data Gap	New Formaldehyde Science
	<p>Additional analyses were performed on the study data obtained from the original study (Zhang et al. 2010) including individual average formaldehyde exposure concentration measurements performed for each exposed worker. The objective was to evaluate hematological parameters and aneuploidy in relation to quantitative exposure measures of formaldehyde. Results showed that differences in white blood cell, granulocyte, platelet, and red blood cell counts were not exposure-dependent. Furthermore, among formaldehyde-exposed workers, no association was observed between individual average formaldehyde exposure estimates and frequency of aneuploidy, suggested by the original study authors to be indicators of myeloid leukemia risk.</p> <p><b>Mundt et al. (2017)</b></p>
<b>D. Dose-Response Assessment</b>	
<p>Independent analysis of the dose-response models is needed to confirm the degree to which the models fit the data appropriately. (NAS, p. 14)</p>	<p>The documentation of the methods applied in the USEPA (2010) IRIS document lacks sufficient detail for duplication of the unit risk estimates provided, even with the availability of the raw data from the <i>Beane Freeman et al. (2010)</i>. This lack of transparency and detail may result in different estimates of unit risks, especially as initial analyses resulted in a lack of a significant dose-response relationship for selected endpoints.</p> <p><b>Van Landingham et al. (2016)</b></p>
<p>BBDR models developed by Conolly and co-workers should be used. (p.58) These models are biologically motivated and mechanistic; requiring that all relevant data be reconciled with the model. (NAS, p.57)</p>	<p>Expansion of the model to incorporate recent data on endogenous levels of formaldehyde is in development. This will incorporate the most recent science to better understand when exogenous formaldehyde exposure appreciably alters normal endogenous formaldehyde concentrations.</p> <p><b>Clewell et al. (in preparation)</b></p>

NAS (2011) Comment / Identified Data Gap	New Formaldehyde Science
Consideration of the use of alternative extrapolation models for the analysis of the cancer data. (NAS, p.14)	Results of the “Bottom-up “ approach indicate that recent top-down risk extrapolations from occupational cohort mortality data for workers exposed to formaldehyde are overly conservative by substantial margins. <b><i>Starr and Swenberg (2013)</i></b>
	Updated “Bottom-Up” risk estimates heighten the marked contrasts that are present between the previous estimates and the corresponding USEPA estimates, with the larger difference for leukemia being due primarily to the significantly improved detection limit for the analytical method used in quantitating DNA adduct numbers. <b><i>Starr and Swenberg (2016)</i></b>
<b><i>E. Methods for Evidence Integration</i></b>	
EPA’s approach to weight of evidence should include “a single integrative step after assessing all of the individual lines of evidence”. Although a synthesis and summary are provided, the process that EPA used to weigh different lines of evidence and how that evidence was integrated into a final conclusion are not apparent in the draft assessment and should be made clear in the final version. (NAS, p. 113)	A hypothesis-based weight-of-evidence (HBWoE) approach was conducted to evaluate the large body of evidence regarding formaldehyde and leukemogenesis, attending to how human, animal, and mode-of-action results inform one another. Upon comparison of alternative proposals regarding what causal processes may have led to the array of observations, it was concluded that the case for a causal association is weak and strains biological plausibility. Instead, apparent association between formaldehyde inhalation and leukemia in some human studies is better interpreted as due to chance or confounding. <b><i>Rhomberg et al. (2011)</i></b>

**Highlights**

- A 2011 NRC report challenged leukemia causation in IRIS Draft Formaldehyde Review
- Studies published since IRIS Draft provide new evidence for evaluating formaldehyde
- Integration of evidence does not support formaldehyde as a cause of leukemia
- Valid hazard classification of formaldehyde has significant regulatory implications

ACCEPTED MANUSCRIPT

Message

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**Subject:** FW: Submission of Letter on Behalf of the ACC Formaldehyde Panel  
**Attachments:** Letter to EPA on NTP 2017 Formaldehyde Study Report - Final.pdf; Attachment 1- NTP Research Report on Absence of Formaldehyde Induced Neoplasia - August 2017.pdf

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**Subject:** Submission of Letter on Behalf of the ACC Formaldehyde Panel

Dear Dr. Bahadori:

Please find attached a letter on behalf of the American Chemistry Council Formaldehyde Panel regarding a recent study report by NTP on formaldehyde.

Kind Regards,

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# NTP

## National Toxicology Program

U.S. Department of Health and Human Services

# NTP RESEARCH REPORT ON

## ABSENCE OF FORMALDEHYDE-INDUCED NEOPLASIA IN TRP53 HAPLOINSUFFICIENT MICE EXPOSED BY INHALATION

NTP RR 3

AUGUST 2017

# **NTP Research Report on Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation**

Research Report 3

National Toxicology Program

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Toxicology Branch  
Division of the National Toxicology Program  
National Institute of Environmental Health Sciences  
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# About this Report

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## Peer Review

The draft research report on the study of formaldehyde exposure to T53 haploinsufficient mice was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies was appropriate and ensured that this NTP Research Report presented the experimental results and conclusions fully and clearly.

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## Abstract

Formaldehyde inhalation is linked to nasal cancer and leukemia in humans. Formaldehyde-induced DNA-protein crosslinks and enhanced cell proliferation are important in the pathogenesis of nasal cancer and, potentially, leukemia. Mutations in the tumor suppressor gene Trp53 have been associated with formaldehyde-induced nasal tumors and might be a key mechanistic event in formaldehyde-induced leukemia. The objective of this study was to evaluate the potential role of the Trp53 gene in formaldehyde-induced nasal carcinogenicity, leukemia or lymphohematopoietic cancer, and potentially other neoplasms in genetically susceptible mice. Male, Trp53 haploinsufficient ( $Trp53^{\pm}$ ) mouse strains (B6.129- $Trp53^{tm1Brd}$  and C3B6.129F1- $Trp53^{tm1Brd}$ ) were exposed to 0-, 7.5- or 15-ppm formaldehyde (25/group) 6 h/d, 5 d/wk for 8 wk, and then held for 32 wk. Blood was collected for hematology, and major tissues and gross lesions were collected for histopathology. The primary formaldehyde-related finding was squamous metaplasia of the respiratory epithelium of the nose. Inhalation of a maximum tolerated dose of formaldehyde caused significant injury to the nasal mucosa and cell proliferation, but did not cause nasal tumors or an increased prevalence of leukemia or lymphohematopoietic cancer in  $Trp53^{\pm}$  mice. All observed neoplasms were considered background lesions for these mouse strains. The results of this short-term carcinogenicity study do not support a role for Trp53 in formaldehyde-induced neoplasia.

Key Words: Formaldehyde, Trp53 mutations,  $Trp53^{\pm}$  mice, nasal cavity, neoplasia, inhalation

## Introduction

Formaldehyde is recognized as a nasal carcinogen in humans<sup>1</sup> and laboratory rats<sup>2-4</sup>. Exposure of rats to formaldehyde results in development of squamous cell carcinoma (SCC) in the anterior and posterior lateral meatus of the nasal cavity<sup>2</sup>. DNA-protein crosslinks (DPC) and cell proliferation are prominent at sites of SCC formation in the rat nasal cavity<sup>4-6</sup>, suggesting that incomplete repair of DPC results in mutations within the proliferating cell population and might lead to neoplasia. Considerable evidence shows that formaldehyde-induced mutations in the tumor suppressor gene *Trp53* are important in the pathogenesis of nasal cancer<sup>7,8</sup>. Mutations in *Trp53* were identified in formaldehyde-induced nasal SCC in rats<sup>9</sup>, and abnormal Trp53 protein was shown to accumulate in the nasal tissue of formaldehyde-exposed rats<sup>10</sup>. Mutations in *Trp53* result in loss of tumor suppression functions including RNA repair, cell cycle arrest, senescence, and apoptosis<sup>11</sup>, resulting in a loss of normal growth control and clonal expansion of mutated cells<sup>12-14</sup>.

Formaldehyde also has been reported to cause myeloid leukemia in humans<sup>15</sup>, although the mechanism is unknown. Mutations in the *Trp53* gene could be important in the pathogenesis of leukemia or lymphohematopoietic cancer. Zhang et al.<sup>16</sup> proposed that hematopoietic stem cells in the nasal epithelium or in circulation undergo formaldehyde-induced mutations that result in loss of *Trp53* and acquisition of the capacity for self-renewal. The *Trp53* gene is involved in regulating the self-renewal of hematopoietic stem cells<sup>17-20</sup>. In addition, disruption of the *Trp53* pathway has been shown to enhance production of induced pluripotent stem cells capable of self-renewal<sup>21</sup>. Acquisition of self-renewal capacity is one of the initial steps in cancer development<sup>22</sup>. Formaldehyde-induced loss of *Trp53* would be rare, however, and indeed an increased incidence of lymphohematopoietic cancer has not been observed in formaldehyde-exposed wild type rats and mice<sup>3</sup>.

Currently no good animal model is available for investigating the mechanism(s) by which formaldehyde causes leukemia. Because considerable evidence suggests a role of *Trp53* in formaldehyde-induced nasal SCC, we evaluated two *Trp53*-haploinsufficient (*Trp53*<sup>±</sup>) mouse strains in this study. We hypothesized that formaldehyde-induced loss of *Trp53* would be increased in *Trp53*<sup>±</sup> mice, resulting in an increased incidence of SCC of the nose and leukemia or lymphohematopoietic cancer, and potentially neoplasms at other sites.

## Materials and Methods

### Animals

Two mouse strains heterozygous for the null and wildtype *Trp53* allele were used to evaluate the potential role of *Trp53* in formaldehyde-induced lymphohematopoietic cancer. The inbred B6.129-*Trp53*<sup>tm1Brd</sup> (Model P53N12-M) mouse strain was selected for this study because of its previous use as a model for lymphohematopoietic tumors in short-term cancer bioassays<sup>23-29</sup>. The C3B6.129F1-*Trp53*<sup>tm1Brd</sup> strain also was selected based on the observation of chemical exposure-dependent lymphohematopoietic tumors of either lymphoid- or myeloid- committed stem cell origin<sup>30,31</sup>. This strain is an outcross between C3H/HeNTac female (Model C3H-F) x B6.129-*Trp53*<sup>tm1Brd</sup> (Model P53N12-M) homozygous *Trp53* null allele male to produce the haploinsufficient F1 progeny. Heterozygotes were selected because they have a lower background incidence of sporadic tumors than the homozygotes, and the latency for sporadically

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occurring tumors is longer than in the homozygote<sup>32; 33</sup>. Although rats appear to be more susceptible than mice to formaldehyde-induced SCC of the nose, a genetically modified rat strain was not available at the time of this study.

Male C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice were 9 weeks old on arrival and male B6.129-*Trp53*<sup>tm1Brd</sup> haploinsufficient mice (Taconic, Hudson, NY) were 8–10 weeks old on arrival. After a 3- (B6.129-*Trp53*<sup>tm1Brd</sup>) to 4-week (C3B6.129F1-*Trp53*<sup>tm1Brd</sup>) quarantine period, mice were weighed and randomly assigned to three exposure groups (Table 1). Five mice per strain were assigned to sentinel groups. All mice were acclimated to the Hazleton 2000 exposure chambers for 3 days prior to exposure. Food (NIH-31) was removed during the 6-hour exposure periods; and water was always available. Body weights were recorded weekly.

### **Formaldehyde Generation and Monitoring**

Nominal chamber concentrations of 7.5-ppm and 15-ppm formaldehyde vapor were generated by heating paraformaldehyde. These concentrations were selected based on reports in the literature and on the results of a 2-week range-finding study in mice. Because cell proliferation plays a key role in formaldehyde-induced neoplasia, exposure concentrations were selected that caused significant injury and cell regeneration in the nasal cavity in the range-finding study. Two 1-liter Woulff bottles containing 200 grams of prilled paraformaldehyde (Sigma Aldrich, St. Louis, MO) were heated on hot plates to 95°C (7.5 ppm) and 120°C (15 ppm). A constant volume (1 liter per minute [LPM]) of breathing-quality air (dried, filtered, and carbon-scrubbed) was delivered to the Woulff bottles. A metered volume of the formaldehyde-enriched headspace was mixed with the chamber supply air stream (500-LPM humidified, filtered, and carbon-scrubbed air). The Hazleton 2000 exposure chamber concentrations were monitored using a Model Z-300XP Formaldehyde Meter (Environmental Sensors, Boca Raton, FL).

### **Animal Exposure**

Mice were individually housed in Hazleton 2000 chambers and exposed to either conditioned air (charcoal and HEPA filtered, temperature and humidity controlled), or to 7.5- or 15-ppm formaldehyde in conditioned air 6 hours/day, 5 days/week, for 8 weeks. This treatment regimen represents a standard 5-day workweek, used for studying chemicals for which exposure is primarily occupational. Exposures of the two strains were staggered by 1 week because of the large numbers of mice to be necropsied. The exposure of the B6.129-*Trp53*<sup>tm1Brd</sup> mice began 1 week after beginning the exposure of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice. Following the 8-week inhalation exposures, mice were transferred to individual polycarbonate cages and monitored for ~32 weeks (until 51–53 weeks of age). Mice were weighed and examined for gross lesions weekly. Mice with large or ulcerated tumors were euthanized and a necropsy conducted.

### **Hematology**

On the day of the scheduled necropsy, whole blood samples were collected under isoflurane anesthesia by means of the retro-orbital venous sinus into tubes containing K<sub>2</sub>EDTA as anticoagulant. The whole blood was analyzed for a complete blood count (CBC) evaluation. CBC end points, including absolute leukocyte (total and differential), erythrocyte (red blood cell [RBC]), reticulocyte (% and absolute [Retics]) and platelet (Plat) counts, hemoglobin (Hgb) concentrations and hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) values, were

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obtained using the ProCyte Dx hematology analyzer (IDEXX Laboratories, Westbrook, ME). A packed cell volume (spun hematocrit) was performed using an Autocrit Ultra 3 micro-hematocrit centrifuge (Becton, Dickinson and Company, Franklin Lakes, NJ). Microscopic evaluation of Romanowsky-stained blood smears was performed to assess leukocyte differential distribution percentages and RBC, WBC, and Plt morphology.

### ***Necropsy***

Mice found moribund during the study and those that survived to the end of the study were euthanized by CO<sub>2</sub> asphyxiation and exsanguination. At necropsy, major organs were weighed and the following tissues, including gross lesions, were collected for pathology evaluations. The distal 1–2 cm of the left femur was sectioned through the frontal plane to include the articular cartilage and articular surface, the femoral condyles with epiphyseal plate, and diaphysis with bone marrow. The left femur was fixed in 10% neutral-buffered formalin (NBF). The nasal cavities were fixed by retrograde infusion of NBF, and then immersed in NBF. The formalin-fixed nasal cavities were decalcified (RDO Rapid Decalcifier; Apex Engineering Products, Aurora, IL) for 12 hours. After decalcification, three separate sections of the nasal cavity were taken at (1) the level of the incisor teeth (Level 1), (2) midway between incisors and first molar (Level 2), and (3) middle of second molar (olfactory region) (Level 3). The remainder of the nasal cavity and turbinates were carefully examined for gross lesions. One-half of the trachea was left attached to the lung. After weighing the lung, the trachea was used to inflate the lungs with fixative. The lungs were trimmed to allow the largest cross-section surface area possible. A transverse section of the larynx was taken at the base of the epiglottis just anterior to the laryngeal saccule. Two sections of liver including transverse sections through the left and median lobes were taken midway along the greatest dimension. A section of gallbladder was included in the section of median lobe. A mid-longitudinal section (left kidney) and a cross section (right kidney) through the entire cortex, pelvis, and medulla of each kidney were collected. Mesenteric, mandibular, mediastinal, and bronchial lymph nodes were collected and placed in cassettes. After formalin fixation, tissues were trimmed and processed, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). A pathologist evaluated the slides, and then the tumor slides were reviewed by four additional pathologists during a Pathology Peer Review.

### ***Immunohistochemistry Staining***

Immunohistochemistry was used to better characterize sarcomas. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. Heat-induced antigen retrieval in citrate buffer (pH 6.0, Biocare Medical, Concord, CA) was done in a Decloaker<sup>®</sup> pressure chamber for 5 minutes at 110°C, followed by 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity. Nonspecific sites were blocked by incubating slides for 20 minutes with 2.5% normal horse serum (Vector, Burlingame, CA). The sections then were then incubated with rabbit monoclonal antimyogenin antibody (Cat# ab124800, Lot# GR155521-1, Abcam, Cambridge, MA) at 1:500 dilution, or a rabbit monoclonal anti-vimentin antibody (Cat# ab92547, lot# GR145336-12, Abcam) at 1:1000 dilution for 1 hour at room temperature. For negative control tissue sections, normal rabbit IgG (Calbiochem<sup>®</sup>, San Diego, CA), diluted to match the protein concentration of the myogenin or vimentin antibodies was utilized. The antigen-antibody complex was detected using ImmPRESS HRP anti-rabbit IgG (Vector) and 3,3'-diaminobenzidine (Dako, Carpinteria, CA). Slides were

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then counterstained with hematoxylin, dehydrated, and cover-slipped. The myogenin slides were stained manually, whereas, the vimentin slides were stained via a Biocare Intellipath™ FLX autostainer (Biocare Medical).

### **Statistical Analyses**

Cochran-Armitage trend tests were used to test for dose-related trends in incidences of neoplasms. Fisher's exact tests (one-sided) were used to compare incidences of neoplasms between each dose group and the control group. Hematology data were analyzed using nonparametric multiple comparison methods<sup>34, 35</sup>.

### **Results**

#### **Survival**

Nine C3B6F1.129-*Trp53*<sup>tm1Brd</sup> animals died prior to the scheduled sacrifice (Table 1). Sixty-five animals of this strain survived to study termination. Sixteen B6.129-*Trp53*<sup>tm1Brd</sup> animals died prior to the time of scheduled sacrifice (Table 1). Most of these animals were euthanized early due to the presence of large, grossly visible tumors. Seventy-five B6.129-*Trp53*<sup>tm1Brd</sup> animals survived to study termination.

#### **Body and Organ Weights**

Body weights of C3B6F1.129-*Trp53*<sup>tm1Brd</sup> mice exposed to 7.5-ppm formaldehyde were significantly less than controls ( $p < 0.05$ ) from week 1 to week 11, and at weeks 37 and 38. Body weights of mice exposed to 15-ppm formaldehyde were significantly less than controls ( $p < 0.05$ ) from week 1 to week 24, and at weeks 37 and 38 (Figure 1). Absolute and relative liver weights were significantly decreased 11% and 8%, respectively, in mice exposed to 15-ppm formaldehyde. Lung, kidney, spleen, and thymus weights of formaldehyde-exposed mice were not significantly different from controls (data not shown).

Body weights of B6.129-*Trp53*<sup>tm1Brd</sup> mice exposed to formaldehyde were not significantly different from controls at any time point (Figure 1). Absolute and relative liver, lung, kidney, spleen, and thymus weights of formaldehyde-exposed B6.129-*Trp53*<sup>tm1Brd</sup> mice were not significantly different from controls (data not shown).

#### **Hematology**

Hematological parameters for formaldehyde-exposed C3B6F1.129-*Trp53*<sup>tm1Brd</sup> (Table 2) and B6.129-*Trp53*<sup>tm1Brd</sup> (Table 3) mice were not significantly different from their respective controls. Neither strain showed any indication of treatment-related hematotoxicity, leukemia, or lymphoma.

#### **Histopathology**

##### *Nasal Cavity*

Formaldehyde exposure-related non-neoplastic lesions were observed primarily in the nasal cavity of C3B6F1.129-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> mice. These nasal lesions were quantitatively and qualitatively similar in both strains. Nasal cavity lesions considered related to

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formaldehyde exposure are summarized in Table 4. The number of animals examined per group includes the early death animals.

The only nasal cavity lesion observed in the control C3B6F1.129-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> animals was minimal hyaline degeneration of the respiratory epithelium, consisting of one or more small foci of epithelial cells containing intra-cytoplasmic accumulation of brightly stained eosinophilic material. This minimal lesion, observed in many of the control animals, is considered a spontaneous background finding.

Squamous metaplasia of the respiratory epithelium was observed in the nasal cavity of many C3B6F1.129-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> mice exposed to 7.5- and 15-ppm formaldehyde. The incidence and average severity (minimal to mild) of squamous metaplasia were greater in the 15-ppm group animals as compared with the 7.5-ppm animals (Table 4). Squamous metaplasia was observed consistently in Level 1, presumably because this level of the nose was exposed to the highest concentration of formaldehyde, and occasionally in Level 2, especially in the 15-ppm exposure groups (Figure 2). This lesion was not observed in Level 3. Squamous metaplasia was observed most commonly on the medial surface of the maxilloturbinates, facing the nasal septum. Other affected sites included the lateral wall, tips of the nasoturbinates, and, occasionally, the dorsal surface of the dorsal meatus. Squamous metaplasia was characterized microscopically by areas of replacement of the normal respiratory epithelium by a thin to occasionally moderately thick layer of stratified squamous epithelium. In some cases, the squamous epithelium was quite thin and was recognized as squamous epithelium by the presence of a layer of thin, laterally flattened cells on the epithelial surface typical of squamous epithelium. Often, a thin to moderately thick layer of keratin, sometimes containing cell debris, was present on the epithelial surface of turbinates in exposed (Figure 3B) but not control (Figure 3A) mice. In a few noses, accumulated keratin was present in the space behind a turbinate scroll, and in some cases filled the space behind the scroll (Figure 3C).

Osteogenesis, proliferation of new bone, was observed in the turbinate bone in a few C3B6.129F1-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> animals exposed to formaldehyde (Table 4). Microscopically, the osteogenesis had a similar appearance in the two strains and in each animal consisted of a small focus of proliferating bone within the lamina propria of the affected turbinate characterized by eosinophilic osteoid containing numerous large plump osteoblast nuclei (Figure 3D).

### *Neoplasms in C3B6.129F1-*Trp53*<sup>tm1Brd</sup> Mice*

Several neoplasms were observed in control and formaldehyde-exposed C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice, and none was considered caused by formaldehyde exposure (Table 5). Three lymphomas were found in the early death C3B6.129F1-*Trp53*<sup>tm1Brd</sup> animals: one in the 7.5-ppm group involving the thymus and two lymph nodes, and the remaining two in the 15-ppm group involving only the thymus. No lymphomas were observed in the control group. The incidences of lymphoma in the formaldehyde-treated groups were not statistically significant ( $p > 0.05$ ) relative to controls. The lymphomas all had a typical and similar morphologic appearance, and consisted of diffuse sheets of relatively large lymphocytes with large, moderately pleomorphic basophilic, granular nuclei with one or more prominent nucleoli and a scant amount of eosinophilic cytoplasm (Supplemental Figure 1).

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Five osteosarcomas were found in bone and skeletal muscle of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice (Table 5). Osteosarcomas were present in two scheduled sacrifice animals (one control and one 7.5-ppm animal), and both had metastasized to the lung. The three remaining osteosarcomas occurred in the early death control animals. Microscopically, the osteosarcomas were discrete masses composed of irregular intersecting trabeculae of eosinophilic osteoid, the matrix material for bone, admixed with variable numbers of highly pleomorphic neoplastic spindle-shaped cells (Figure 4A). Although osteosarcomas are considered to originate from bone, three osteosarcomas (from the early death control animals) had skeletal muscle attached to them but no bone. We assumed they had originated in bone and had invaded adjacent skeletal muscle, but that the bony origins of these neoplasms were not included in the microscopic sections. Osteosarcomas stained positively for vimentin, an intermediate filament typically present in cytoplasm of non-epithelial cells (Figure 4B), and stained negatively for smooth muscle actin, myogenin, and F4/80.

In addition to osteosarcomas, an undifferentiated sarcoma was also observed in the skeletal muscle of a 7.5-ppm C3B6.129F1-*Trp53*<sup>tm1Brd</sup> animal. This neoplasm was a large, discrete mass consisting of numerous large, round to ovoid to elongated cells with abundant eosinophilic cytoplasm and small basophilic nuclei. Hepatocellular adenoma and carcinoma, histiocytic sarcoma, and hemangiosarcoma also were observed in some animals, but the incidences were not significantly increased or concentration related (Table 5).

Osteosarcomas in two control mice and a hemangiosarcoma in one 7.5-ppm animal were in contact with or had enveloped the microchip transponders used for animal identification. Sarcomas, osteosarcomas, and histiocytic sarcoma in eight C3B6.129F1-*Trp53*<sup>tm1Brd</sup> animals were located remotely from the microchips.

### *Neoplasms in B6.129-Trp53*<sup>tm1Brd</sup> Mice

A variety of observed neoplasms in formaldehyde-exposed B6.129-*Trp53*<sup>tm1Brd</sup> mice were not considered chemical related (Table 6). Lymphomas were present in two formaldehyde-exposed B6.129-*Trp53*<sup>tm1Brd</sup> animals, one in the 7.5-ppm group and one in the 15-ppm group. The lymphoma in the 7.5-ppm animal, which appeared to have originated in the thymus, had spread widely, being present in nearly every tissue examined. In contrast, the lymphoma in the 15-ppm animal appeared to have originated in the mesenteric lymph node with a small amount of involvement of the bronchial lymph node. The lymphomas appeared morphologically similar to one another and to the lymphomas observed in the C3B6.129F1-*Trp53*<sup>tm1Brd</sup> animals (Supplemental Figure 1).

Skeletal muscle rhabdomyosarcomas were observed in five formaldehyde-exposed B6.129-*Trp53*<sup>tm1Brd</sup> animals, one in the 7.5-ppm group and four in the 15-ppm group (includes early death animals) (Table 6). Rhabdomyosarcomas were not observed in B6.129-*Trp53*<sup>tm1Brd</sup> control animals. Although a statistically significant trend ( $p=0.042$ ) was found, the incidence of rhabdomyosarcomas in the 15-ppm group was not statistically significant ( $p=0.133$ ) relative to controls. Microscopically, the neoplasms were large masses composed of solid foci and interlacing bands of neoplastic cells generally admixed with varying amounts of dense fibrous tissue stroma (Figure 4C). The neoplasms were composed primarily of variably sized pleomorphic polygonal to fusiform cells, with large ovoid to fusiform, moderately basophilic to vesicular nuclei with one to several prominent nucleoli and small to moderate and occasionally large amounts of eosinophilic cytoplasm with indistinct borders. Few to numerous mitotic

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figures were present, and varying numbers of large multinucleated cells resembling rhabdomyoblasts were observed. Cross striations were not observed; however, the tumors stained positively for vimentin, a marker for cells of mesenchymal origin including skeletal muscle, and myogenin, a nuclear transcription factor essential for skeletal muscle development and repair, and a specific marker for skeletal muscle (Figure 4D).

Various other neoplasms occurred at low incidences in control and formaldehyde-exposed B6.129-*Trp53*<sup>tm1Brd</sup> animals, all of which were considered background lesions unrelated to formaldehyde exposure (Table 6). Microchip transponders were found in contact with, or enveloped by, tumors in five B6.129-*Trp53*<sup>tm1Brd</sup> animals exposed to formaldehyde. Sarcomas in 10 B6.129-*Trp53*<sup>tm1Brd</sup> animals were not associated with microchip transponders.

### Discussion

Formaldehyde-induced injury to the nasal epithelium, regenerative proliferation, and mutations in these proliferating cells are key steps in the pathogenesis of formaldehyde-induced nasal SCC<sup>4,6</sup>. Mutations in the tumor suppressor gene *Trp53* have been detected in formaldehyde-induced nasal tumors and in preneoplastic hyperkeratotic plaques, indicating that *Trp53* mutations are involved in the pathogenesis of SCC<sup>9,10</sup>. Based on these data, *Trp53*<sup>±</sup> mice were expected to be highly susceptible to formaldehyde-induced nasal SCC, and possibly leukemia and other neoplasms. Under the conditions of this short-term study, however, formaldehyde exposure did not result in SCC of the nasal cavity or in an increased incidence of leukemia or lymphohematopoietic neoplasms in two genetically susceptible *Trp53*<sup>±</sup> mouse strains. Formaldehyde-induced loss heterozygosity (LOH) in *Trp53* and loss of tumor suppressor function may have been insufficient to induce neoplasia. Although mutations to *Trp53* can result in a dysfunctional protein, some mutations to *Trp53* can result in a gain of function (GOF) that promotes tumor development<sup>36,37</sup>. Formaldehyde-induced mutations of *Trp53* that result in GOF may be necessary for induction of neoplasia.

Although rats are more susceptible than mice to formaldehyde-induced nasal tumors<sup>3</sup>, a *Trp53*<sup>±</sup> rat strain was not available at the time of this study. Mice are reportedly less susceptible than rats because of their greater ability to reduce their minute ventilation upon repeated formaldehyde exposures<sup>38,39</sup>, thereby reducing tissue damage and cell turnover in the nasal mucosa. Spontaneous SCC of the nasal cavity is extremely rare in rats and mice. Tumors of the nasal cavity have not been observed in NTP historical controls for B6C3F1/N or B6.129-*Trp53*<sup>tm1Brd</sup> mice. Background nasal tumor incidences for the C3B6.129F1-*Trp53*<sup>tm1Brd</sup> were not available. Induction of SCC in wild type mice by formaldehyde is also extremely rare. Exposure of C57BL/6 x C3HF1 mice to 14.3-ppm formaldehyde for 2 years followed by a 6-month holding period resulted in only two mice with nasal tumors<sup>3</sup>.

In this study, mice were exposed to concentrations of formaldehyde that caused significant cell injury, inflammation, and regeneration in the nasal mucosa, which are recognized as key events in nasal SCC development<sup>5</sup>. Although significant regenerative cell proliferation and squamous metaplasia were observed in exposed mice, a higher formaldehyde concentration, a longer exposure duration, or a longer holding period might be necessary for accumulation of genetic alterations in *Trp53* and development of SCC in mice. The severity of nasal lesions in both mouse strains, the significantly reduced body weights of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice, and the lack of body weight gain in both strains, however, indicated that exposure to 15 ppm for 8 weeks

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represented a maximum tolerated dose. An 8-week exposure duration was considered sufficient because the doubling time for HSPCs is reported to be between 2 and 4 weeks with the entire HSPC pool turning over every 8 weeks<sup>40; 41</sup>. Although exposed mice could have been held longer, 50 weeks of age was selected because, at approximately 70 weeks of age, about one-half of Trp53<sup>±</sup> mice reportedly develop background tumors<sup>33</sup>.

Formaldehyde exposure did not significantly increase the incidence of leukemia or lymphohematopoietic neoplasms in either B6.129-*Trp53*<sup>tm1Brd</sup> or C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice. The formaldehyde exposure concentrations were observed to cause disruption of the nasal epithelium, potentially allowing exposure of the underlying nasal cavity stem cells and circulating hematopoietic stem cells in the nasal vasculature. Maximal exposure of stem cells would be expected to occur following disruption (ulceration, necrosis) of the epithelium. As noted in this study, however, the accessibility of the nasal stem cells to inhaled formaldehyde could decrease as the damaged nasal epithelium is rapidly replaced by the more resistant squamous epithelium. In addition, the squamous epithelium produced multiple layers of keratin that might also protect underlying cells from inhaled formaldehyde. Whether a similar phenomenon occurs in exposed rats is unclear. Lymphoma was observed in several formaldehyde-exposed mice and was absent in control groups for both mouse strains. The low incidence of lymphoma in formaldehyde-exposed mice, however, was not statistically significant. Lymphoma was not considered exposure related because of the low incidence, the lack of statistical significance, and because lymphoma is one of the most common spontaneous tumors reported in Trp53<sup>±</sup> mice<sup>32; 42</sup>.

Sarcomas were the most prevalent tumor observed in B6.129-*Trp53*<sup>tm1Brd</sup> mice and were the most common cause of early deaths due to the rapid growth of these tumors. The use of subcutaneous microchip transponders for animal identification might have influenced the incidence of sarcomas in B6.129-*Trp53*<sup>tm1Brd</sup>. Sarcomas can occur in B6.129-*Trp53*<sup>tm1Brd</sup> mice in association with subcutaneous microchips but can also occur spontaneously and in reaction to other treatments<sup>43</sup>. Although the mechanism for a synergistic or additive effect of subcutaneous microchip implantation and formaldehyde exposure on skeletal muscle sarcoma development in B6.129-*Trp53*<sup>tm1Brd</sup> is not clear, evidence is insufficient to rule out such an effect.

Osteosarcoma was a common spontaneous lesion in C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice, occurring in about 15% of controls and in only one animal exposed to formaldehyde (7.5 ppm).

Osteosarcomas are generally uncommon neoplasms in mice but appear to be more common in Trp53<sup>±</sup> mice. Microchips were found within or in contact with tumors in two of the four control mice with osteosarcoma, and in one mouse in the 7.5-ppm group, the microchip was in contact with a hemangiosarcoma. As noted with the skeletal muscle sarcomas in B6.129-*Trp53*<sup>tm1Brd</sup> mice, immunostaining of osteosarcomas associated with, or remote from, microchips showed no differences.

### Conclusion

Under the conditions of this study, inhalation exposure to a maximum tolerated dose of formaldehyde did not cause an increased incidence of nasal SCC, leukemia, or lymphohematopoietic tumors in Trp53<sup>±</sup> mice. Both mouse strains developed a variety of neoplasms, but all were considered spontaneous background lesions and not a result of

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formaldehyde exposure. The results of this short-term carcinogenicity study do not support a role for Trp53 in formaldehyde-induced neoplasia.

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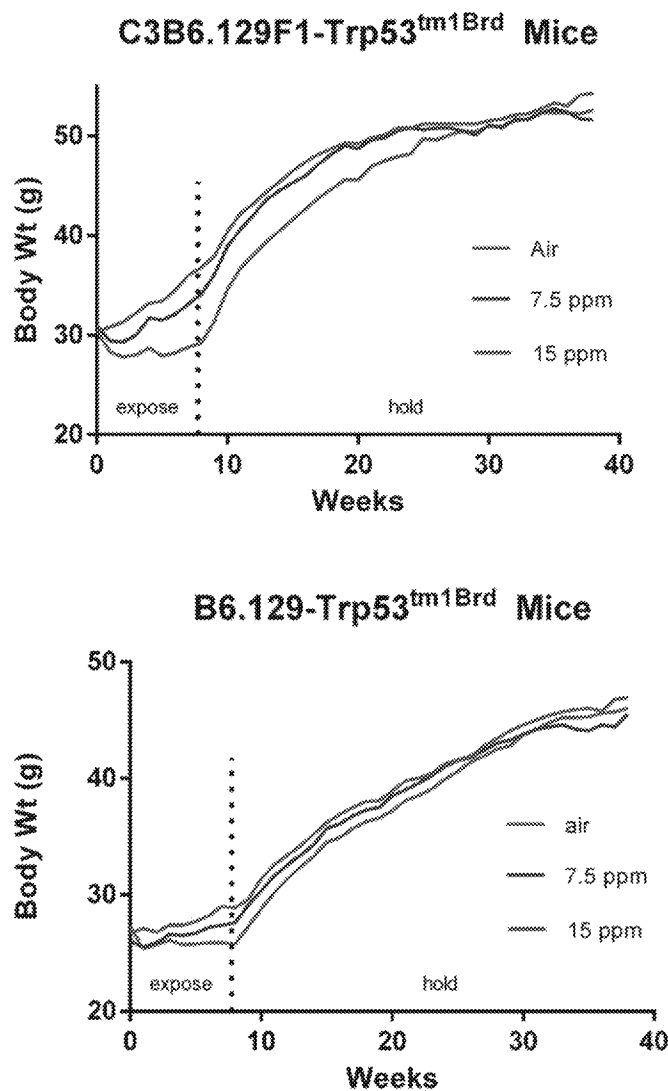
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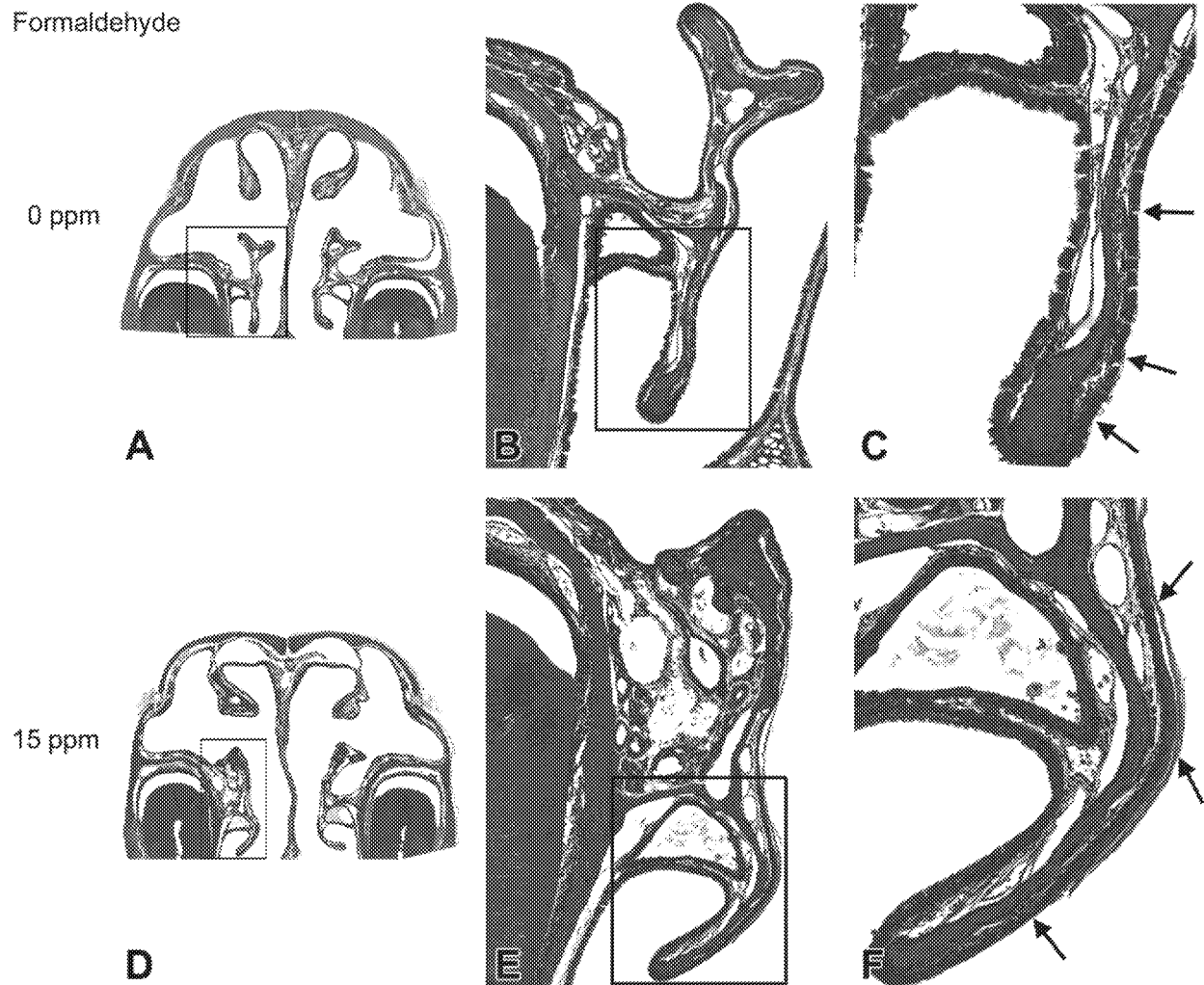
# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation



**Figure 1. Body Weights of Trp53<sup>±</sup> Mice Exposed to Formaldehyde**

C3B6.129F1-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> mice were exposed to formaldehyde at 0 (air), 7.5, or 15 ppm for 8 weeks, and then held for 32 weeks without exposure. (A) Body weights of C3B6F1.129-*Trp53*<sup>tm1Brd</sup> mice exposed to 7.5-ppm formaldehyde were significantly less than controls ( $p < 0.05$ ) from week 1 to week 11 and at weeks 37 and 38. Body weights of mice exposed to 15-ppm formaldehyde were significantly less than controls ( $p < 0.05$ ) from week 1 to week 24 and at weeks 37 and 38. (B) Body weights of B6.129-*Trp53*<sup>tm1Brd</sup> mice exposed to formaldehyde were not significantly different from controls at any time point.

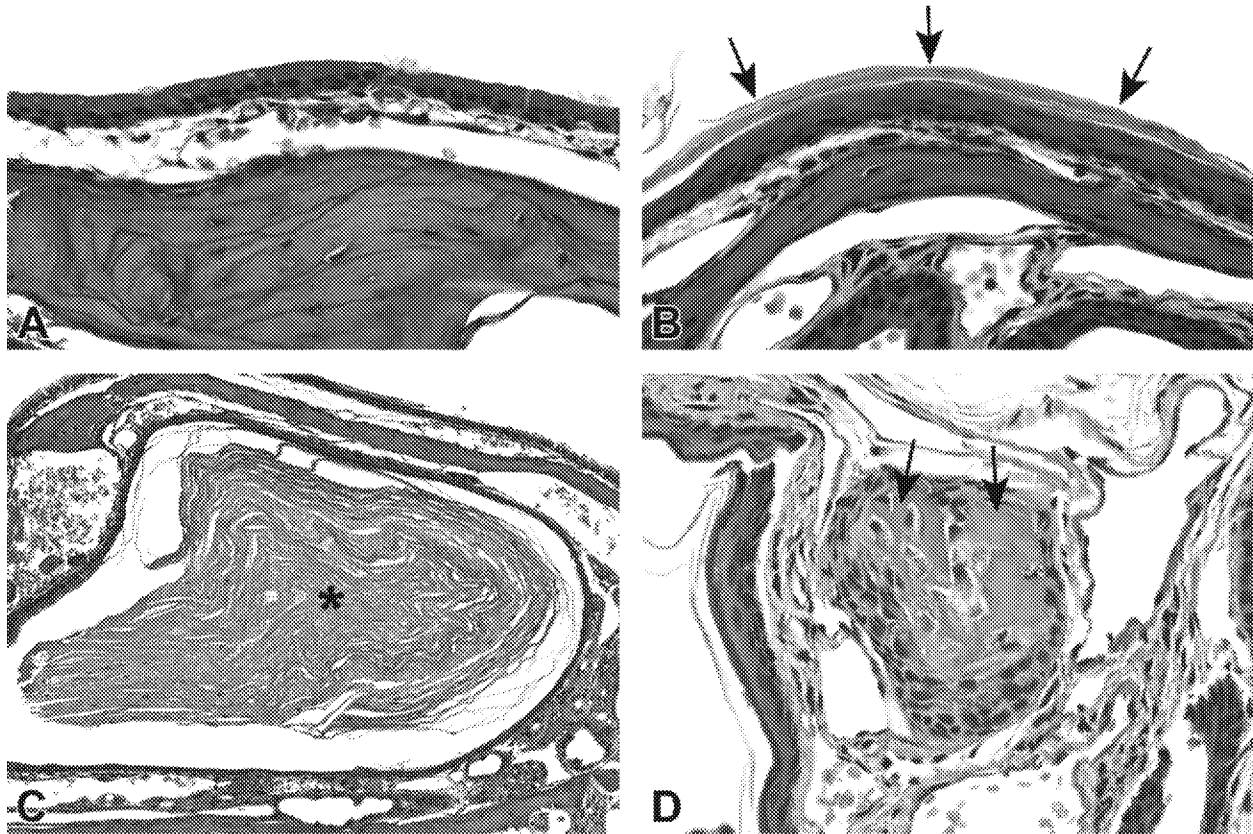
# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation



**Figure 2. Squamous Metaplasia of the Nasal Cavity in Mice Exposed to Formaldehyde**

(A) A low magnification showing the location of the maxilloturbinates (within the box) and the nasoturbinates located dorsally and attached to the roof of the nasal cavity on either side of the nasal septum, Level 1. (B and C) Higher magnifications of the maxilloturbinate (within the box) showing the normal respiratory epithelium covering the turbinate bones. The normal respiratory epithelium in this area consists of a single layer of ciliated, columnar, and non-ciliated cuboidal cells (arrows in C). (D, E, and F) Level I section of the nasal cavity from a high-dose (15-ppm) animal. As in A, panel D is a low magnification showing the location of the maxilloturbinates (within the box) and the nasoturbinates dorsally. E and F are higher magnifications of the affected turbinate epithelium (within the box). The normal respiratory epithelium has been replaced by keratinized, stratified squamous epithelium (arrows in panel F).

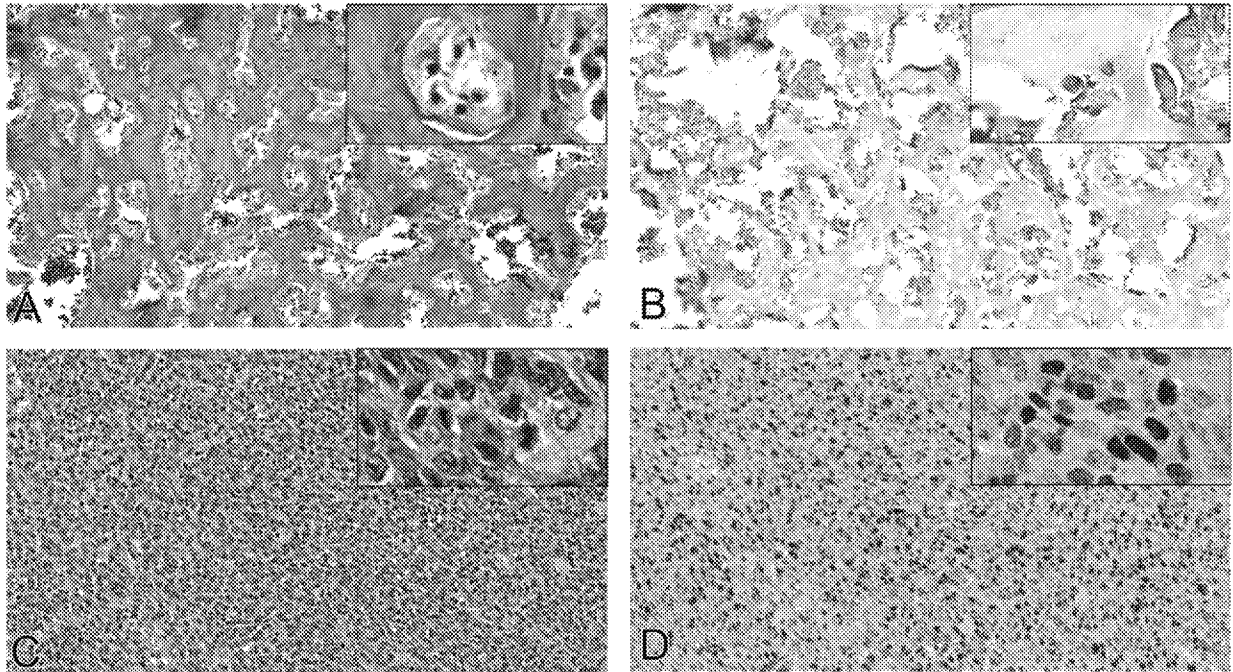
# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation



**Figure 3. Keratin Accumulation and Osteogenesis in Nasal Cavity of Mice Exposed to Formaldehyde**

C3B6.129F1-*Trp53*<sup>tm1Brd</sup> and B6.129-*Trp53*<sup>tm1Brd</sup> mice were exposed to formaldehyde at 0 (air), 7.5, or 15 ppm for 8 weeks, and then held for 32 weeks without exposure. Significant lesions were still present in the nasal cavity of both mouse strains when examined 32 weeks after formaldehyde exposure. (A) Normal appearing respiratory epithelium on the nasal turbinate of an air-exposed control C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mouse. H&E, 60x. (B) Squamous metaplasia of respiratory epithelium on the nasal turbinate of a C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mouse exposed to 15-ppm formaldehyde. Note multiple layers of keratin (arrows). H&E, 60x. (C) In some animals exposed to 15-ppm formaldehyde, accumulated keratin was present in the space behind a turbinate scroll and, in some cases, filled the space behind the scroll (asterisk). H&E, 20x. (D) Minimal osteogenesis of the nasoturbinate was present in a few mice exposed to 15-ppm formaldehyde. The lesion consisted of a small focus within the lamina propria consisting of eosinophilic osteoid containing numerous large plump osteoblast nuclei (arrows). H&E, 60x.

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**Figure 4. Neoplastic Lesions in  $Trp53^{\pm}$  Mice Exposed to Formaldehyde**

Histological appearance (A, C); hematoxylin and eosin, and immunohistochemical staining with vimentin (B) and myogenin (D) of tumors in C3B6.129F1- $Trp53^{tm1Brd}$  mice and B6.129- $Trp53^{tm1Brd}$  mice. (A) Osteosarcoma in a C3B6.129F1- $Trp53^{tm1Brd}$  mouse, characterized by formation of irregular bone trabeculae and adjacent highly pleomorphic neoplastic spindle-shaped cells. Inset: Higher magnification of pleomorphic neoplastic cell types adjacent to bone trabeculae. (B) Vimentin-stained section of osteosarcoma shown in (A). Note pleomorphic neoplastic cells with cytoplasmic expression of vimentin, an intermediate filament typically present in non-epithelial cells. Inset: Higher magnification of pleomorphic neoplastic cell types with positively staining cytoplasm. (C) Rhabdomyosarcoma in a B6.129- $Trp53^{tm1Brd}$  mouse, characterized by pleomorphic cells with varying amounts of cytoplasm and oval-to-elongated nuclei. Inset: Higher magnification of tumor cells showing oval-to-elongated nuclei, abundant cytoplasm, and mitotic activity. (D) Myogenin-stained section of rhabdomyosarcoma shown in (C). Note variably sized pleomorphic tumor cells with nuclear expression of myogenin, a transcription factor essential for skeletal muscle development and repair. Inset: Higher magnification of pleomorphic tumor cells showing positively staining oval and elongated nuclei.

# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation

**Table 1. Mortality in Trp53<sup>±</sup> Mice Exposed to Formaldehyde**

Formaldehyde Concentration	0 ppm	7.5 ppm	15 ppm
<b>C3B6.129F1-Trp53<sup>tm1Brd</sup> Mice</b>			
<b>Animals/group:</b>	25	24	25
<b>Early Deaths:</b>	4 (16%)	3 (11%)	2 (8%)
<b>Cause of Death:</b>			
Lymphoma	0	1	2
Osteosarcoma	3	0	0
Sarcoma	0	1	0
Carcinoma NOS <sup>a</sup> metastatic <sup>b</sup>	0	1	0
Undetermined	1	0	0
<b>B6.129-Trp53<sup>tm1Brd</sup> Mice</b>			
<b>Animals/group:</b>	25	31	35
<b>Early Deaths:</b>	3 (12%)	4 (13%)	9 (26%)
<b>Cause of Death:</b>			
Rhabdomyosarcoma	0	1	3
Sarcoma	1	2	1
Osteosarcoma	1	0	0
Leiomyosarcoma	0	0	1
Carcinosarcoma	0	0	1
Histiocytic sarcoma	0	0	1
Ulcerative dermatitis	0	0	1
Nephropathy	0	0	1
Undetermined	1	1	0

Formaldehyde exposure had no statistically significant effect on survival of either mouse strain. Nine *C3B6.129F1-Trp53<sup>tm1Brd</sup>* mice died or were euthanized early primarily due to the presence of large or ulcerated osteosarcomas. Most early deaths of *B6.129-Trp53<sup>tm1Brd</sup>* mice (16) were due to the presence of large sarcomas and rhabdomyosarcomas.

<sup>a</sup> NOS - not otherwise specified.

<sup>b</sup> Carcinoma in lung, primary site unknown.

# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation

**Table 2. Hematological Parameters of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> Mice Exposed to Formaldehyde**

Erythron							
Formaldehyde Concentration	RBC 10 <sup>6</sup> /ml	HgB g/dl	Hct %	MCV fL	MCH pg	MCHC g/dl	Retics 10 <sup>6</sup> /μl
0 ppm	9.45 ± 0.52	14.7 ± 0.7	46.7 ± 2.2	49.5 ± 0.7	15.6 ± 0.3	31.5 ± 0.4	0.29 ± 0.05
7.5 ppm	9.29 ± 1.08	14.3 ± 1.5	45.4 ± 4.6	49.0 ± 1.5	15.4 ± 0.5	31.5 ± 0.4	0.32 ± 0.12
15 ppm	9.44 ± 0.51	14.7 ± 0.8	46.2 ± 2.2	49.0 ± 0.8	15.5 ± 0.2	31.7 ± 0.5	0.31 ± 0.07
Leukon							
Formaldehyde Concentration	WBC 10 <sup>3</sup> /μl	Neut 10 <sup>3</sup> /μl	Lymph 10 <sup>3</sup> /μl	Mono 10 <sup>3</sup> /μl	Eos 10 <sup>3</sup> /μl	Plats 10 <sup>3</sup> /μl	
0 ppm	5.07 ± 1.40	1.05 ± 1.09	3.67 ± 0.86	0.20 ± 0.11	0.13 ± 0.10	1312 ± 214	
7.5 ppm	4.93 ± 1.70	1.13 ± 0.94	3.45 ± 0.79	0.21 ± 0.14	0.12 ± 0.09	1283 ± 200	
15 ppm	4.37 ± 0.79	0.80 ± 0.26	3.23 ± 0.57	0.22 ± 0.11	0.12 ± 0.04	1209 ± 121	

Formaldehyde exposure had no significant effect on any of the hematological parameters of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> mice.

**Table 3. Hematological Parameters of B6.129-*Trp53*<sup>tm1Brd</sup> Mice Exposed to Formaldehyde**

Erythron							
Formaldehyde Concentration	RBC 10 <sup>6</sup> /ml	HgB g/dl	Hct %	MCV fL	MCH pg	MCHC g/dl	Retics 10 <sup>6</sup> /μl
0 ppm	9.62 ± 0.33	14.0 ± 0.5	46.4 ± 1.5	48.3 ± 0.9	14.6 ± 0.3	30.2 ± 0.4	0.31 ± 0.03
7.5 ppm	9.34 ± 1.09	13.6 ± 1.9	45.4 ± 4.6	48.8 ± 2.2	14.5 ± 0.8	29.7 ± 2.1	0.39 ± 0.31
15 ppm	9.38 ± 0.62	13.7 ± 0.8	45.3 ± 2.8	48.3 ± 0.9	14.6 ± 0.3	30.3 ± 0.6	0.30 ± 0.03
Leukon							
Formaldehyde Concentration	WBC 10 <sup>3</sup> /μl	Neut 10 <sup>3</sup> /μl	Lymph 10 <sup>3</sup> /μl	Mono 10 <sup>3</sup> /μl	Eos 10 <sup>3</sup> /μl	Plats 10 <sup>3</sup> /μl	
0 ppm	5.4 ± 1.8	0.60 ± 1.50	4.44 ± 0.77	0.192 ± 0.07	0.127 ± 0.03	1409 ± 182	
7.5 ppm	5.9 ± 1.9	0.97 ± 1.49	4.54 ± 0.92	0.220 ± 0.08	0.125 ± 0.04	1350 ± 292	
15 ppm	5.3 ± 1.3	0.62 ± 0.29	4.37 ± 1.12	0.172 ± 0.07	0.141 ± 0.06	1344 ± 283	

Formaldehyde exposure had no significant effect on any of the hematological parameters of B6.129-*Trp53*<sup>tm1Brd</sup> mice.

# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation

**Table 4. Nasal Lesions in Trp53<sup>±</sup> Mice Exposed to Formaldehyde**

Formaldehyde Concentration	0 ppm	7.5 ppm	15 ppm
<b>C3B6.129F1-<i>Trp53</i><sup>tm1Brd</sup> Mice</b>			
Respiratory Epithelium			
Squamous metaplasia	0/21 <sup>a</sup>	14/21 (1.2) <sup>b</sup>	22/23 (1.5)
Hyperplasia	0/21	0/21	1/23 (1.0)
Turbinate			
Osteogenesis	0/21	0/21	3/23 (3.0)
<b>B6.129-<i>Trp53</i><sup>tm1Brd</sup> Mice</b>			
Respiratory Epithelium			
Squamous metaplasia	0/22	13/27 (1.0)	17/26 (1.5)
Turbinate			
Osteogenesis	0/22	1/27 (1.0)	1/26 (1.0)

Mice were exposed to conditioned air, 7.5 or 15 ppm formaldehyde 6hr/d, 5d/week for 8 weeks and then held without further exposure for 32 weeks.

<sup>a</sup> Number of mice with lesion/number examined.

<sup>b</sup> Average severity score based upon 1=minimal, 2=mild, 3=moderate, 4=marked.

**Table 5. Neoplasms in C3B6.129F1-*Trp53*<sup>tm1Brd</sup> Mice Exposed to Formaldehyde**

Formaldehyde Concentration	0 ppm	7.5 ppm	15 ppm
<b>Number examined:</b>	<b>25</b>	<b>24</b>	<b>25</b>
Osteosarcoma	4 <sup>a</sup> (16%) <sup>b</sup>	1 (4.2%)	0
Sarcoma, NOS <sup>c</sup> , stomach/rib cage	0	1 (4.2%)	0
Sarcoma, NOS, subcutis	1 (4%)	1 (4.2%) <sup>d</sup>	0
Sarcoma, NOS <sup>e</sup>	0	0	1 (4%)
Sarcoma, NOS, Harderian gland	0	1 (4.2%)	0
Hepatocellular adenoma	5 (20%)	4 (16.7%)	1 (4%)
Hepatocellular carcinoma	0	2 (8.3%)	0
Alveolar/bronchiolar carcinoma	1 (4%)	0	0
Hemangiosarcoma, subcutis	0	1 (4.2%) <sup>d</sup>	0
Lymphoma	0	1 (4.2%)	2 (8%)

Mice were exposed to conditioned air, 7.5 or 15 ppm formaldehyde 6hr/d, 5d/week for 8 weeks and then held without further exposure for 32 weeks.

<sup>a</sup> Number of mice with lesion (includes animals that died early).

<sup>b</sup> Percentage of animals with lesion.

<sup>c</sup> Not otherwise specified.

<sup>d</sup> Sarcoma and hemangiosarcoma present in same animal.

<sup>e</sup> Tissue undetermined.

# Absence of Formaldehyde-Induced Neoplasia in Trp53 Haploinsufficient Mice Exposed by Inhalation

**Table 6. Neoplasms in B6.129-*Trp53*<sup>tm1Brd</sup> Mice Exposed to Formaldehyde**

Formaldehyde Concentration	0 ppm	7.5 ppm	15 ppm
<b>Number Examined:</b>	<b>25</b>	<b>31</b>	<b>35</b>
Osteosarcoma	1 <sup>a</sup> (4.0%) <sup>b</sup>	0	0
Hepatocellular adenoma	2 (8.0%)	0	0
Alveolar/bronchiolar adenoma	1 (4.0%)	0	0
Histiocytic sarcoma	0	1 (3.2%)	2 (5.7%)
Lymphoma	0	1 (3.2%)	1 (2.9%)
Rhabdomyosarcoma	0*	1 (3.2%)	4 (11.4%)
Leiomyosarcoma, subcutis	0	0	1 (2.9%)
Carcinosarcoma, subcutis	0	0	1 (2.9%)
Sarcoma, NOS <sup>c</sup> , subcutis	1 (4.0%)	3 (9.7%)	1 (2.9%)

Mice were exposed to conditioned air, 7.5 or 15 ppm formaldehyde 6hr/d, 5d/week for 8 weeks and then held without further exposure for 32 weeks.

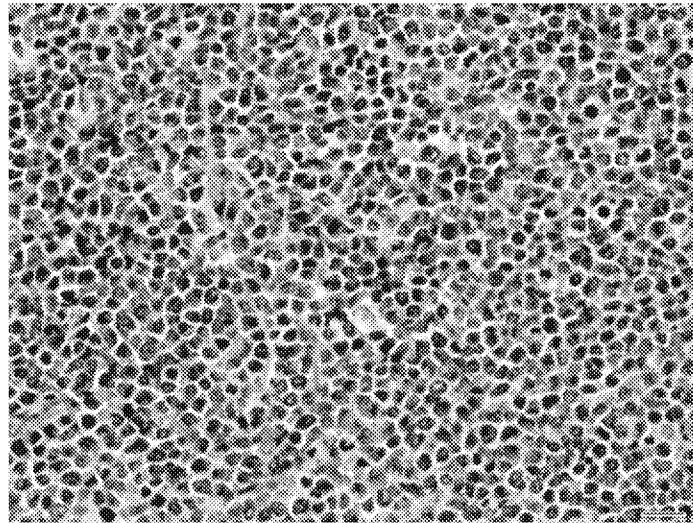
<sup>a</sup> Number of mice with lesion (includes animals that died early).

<sup>b</sup> Percentage of animals with lesion.

<sup>c</sup> Not otherwise specified.

\* Significant trend (p<0.05).

**Supplemental Materials**



**Supplemental Figure 1. Thymus, Cortex, Lymphoma of C3B6.129F1-*Trp53*<sup>tm1Brd</sup> Mice Exposed to Formaldehyde**

The lymphomas appeared as diffuse sheets of relatively large lymphocytes with large, moderately pleomorphic basophilic, granular nuclei with one or more prominent nucleoli and a scant amount of eosinophilic cytoplasm. H&E, 40X.